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Notes:

Inhibition of translation by poliovirus: Inactivation of a specific initiation factor

(translational control/eukaryotic initiation factor-4B/*in vitro* translation/vesicular stomatitis virus)

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ABSTRACT Translation of vesicular stomatitis virus (VSV) mRNA, like host mRNA translation, is inhibited in cells infected with poliovirus. To study the mechanism of poliovirus-induced inhibition of protein synthesis, we prepared extracts from poliovirus-infected and uninfected HeLa cells. Poliovirus mRNA was translated in lysates from both infected and uninfected cells, while VSV mRNA was translated only in the lysate from uninfected cells. Addition of purified translation initiation factors to the extract from infected cells showed that one factor, eIF-4B, could restore VSV mRNA translation in the infected lysate, but did not increase poliovirus mRNA translation. Further experiments involving translation of VSV mRNA in mixed extracts from poliovirus-infected and uninfected cells showed (i) that there was not an excess of an inhibitor of VSV mRNA translation in the infected lysate, but (ii) that an activity that caused a slow inactivation of eIF-4B was present in the infected lysate. Inactivation of eIF-4B appears to be the mechanism by which poliovirus infection causes a selective inhibition of translation.

Inhibition of host protein synthesis occurring after virus infection has been demonstrated in many eukaryotic virus-host systems (1). The best studied case of inhibition of protein synthesis is that caused by picornaviruses such as poliovirus, but the mechanism that allows discrimination between host and viral mRNA translation has not been elucidated. Infection by poliovirus results in extensive inhibition of host protein synthesis at the initiation step (2, 3). During poliovirus infection the host mRNAs are not degraded (2, 3) and their capping, methylation, and polyadenylation are not affected (4). Furthermore, translation of vesicular stomatitis virus (VSV) mRNAs is prevented when VSV-infected cells are superinfected with poliovirus (5, 6). VSV mRNA synthesis continues normally in the superinfected cells and the untranslated VSV mRNA can be translated *in vitro* after purification from the infected cell (6). Also, the kinetics of poliovirus inhibition of host and VSV protein synthesis are identical, suggesting that they occur by the same mechanism (6).

In this study we have used translation of VSV mRNA in extracts derived from poliovirus-infected and uninfected HeLa cells to study the mechanism of translation inhibition by poliovirus. VSV directs the synthesis of five mRNAs that encode the five viral proteins (7, 8). These mRNAs, like cellular mRNAs, have capped and methylated 5' termini that are important in ribosome recognition (9). In contrast, poliovirus mRNA has a 5' terminus of pUp (10, 11). The evidence presented here indicates that loss of a single initiation factor activity (eIF-4B) can explain the inhibition of VSV and host mRNA translation by poliovirus. Other work has indicated that this factor interacts with the 5' cap structure on mRNA (12). Thus, poliovirus mRNA may bypass the cap-dependent recognition

mechanism and also inactivate an initiation factor involved in recognition of capped mRNA.

A recent study (13) has described the roles of seven protein factors in assembly of the eukaryotic translation initiation complex. Briefly, eIF-2 and GTP are required for formation of the 40S-Met-tRNA_f complex. ATP and five factors, eIF-3, eIF-4A, eIF-4B, eIF-4C, and probably eIF-1 are involved in mRNA binding to the 40S-Met-tRNA_f complex. Factor eIF-5 and GTP promote joining of the 60S subunit to form the 80S-Met-tRNA_f-mRNA complex.

MATERIALS AND METHODS

Poliovirus Infection and Preparation of Translation Systems. The growth of HeLa cells in suspension in Joklik's modified minimal essential medium and infection by type 1 poliovirus were as described (14) except that fetal calf serum was substituted for horse serum. For infection, 4×10^8 HeLa cells were harvested, washed once, and resuspended at 4×10^8 cells per ml in minimal essential medium containing poliovirus at a multiplicity of 20. After adsorption at room temperature for 30 min, cells were diluted to 4×10^6 /ml in minimal essential medium plus 5% fetal calf serum. Except as indicated, infected or mock-infected cells were harvested 3 hr after infection and the cell-free translation system was prepared as described (15) with the following modifications. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer was used instead of Tris-HCl in the isotonic buffer, and cells were resuspended in 1.5 vol instead of in 3 vol of buffer. Cells were left at 0° for 2 min before homogenization. After homogenization, 5/18 vol of 10× incubation buffer was added. Preincubation for 45 min was as described (15), followed by dialysis for 2 hr against 100 vol 10 mM Hepes, pH 7.5/90 mM KCl/1.5 mM Mg(OAc)₂/7 mM 2-mercaptoethanol. Micrococcal nuclease treatment (75 units/ml of extract) was for 15 min at 20° as described (16). Small samples of the extract were stored at -80°.

Cell-Free Translation. *In vitro* translation reaction mixtures (25 μ l) contained 10 μ l of lysate and the following additions: 20 μ M of each of 19 amino acids (minus methionine), 0.8 mM ATP, 20 μ M GTP, 6.4 mM creatine phosphate, 0.2 μ g of creatine phosphokinase (Calbiochem), 1.6 mM dithiothreitol, 14.2 mM Hepes (pH 7.2), 0.2 mM spermidine, 80 mM KOAc, 0.65 mM Mg(OAc)₂, 5 μ Ci of [³⁵S]methionine (Amersham, Inc., 300 Ci/mmol), and 1 μ g each of VSV mRNA, rabbit globin mRNA, or poliovirus mRNA. Reactions were for 1 hr at 37° followed by addition of RNase A (final concentration of 100 units/ml) and further incubation for 5 min. Samples were precipitated with acetone, heated to 90° for 2 min in sample buffer, and

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Abbreviations: VSV, vesicular stomatitis virus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; eIF, eukaryotic initiation factor.

subjected to gel electrophoresis by the procedure of Laemmli (17).

Purification of mRNAs and Protein Synthesis Initiation Factors. RNA from VSV-infected cells was prepared as described (18) except that oligo(dT)-cellulose chromatography was omitted. VSV mRNA was assumed to be 2% of the total RNA by weight. Poliovirus RNA was purified by phenol extraction of virions, followed by ethanol precipitation. Rabbit globin mRNA was purified as described (19). Removal of the protein from the 5' end of poliovirus virion RNA (20, 21) occurs rapidly in this translation system (V. Ambros, R. Pettersson, and D. Baltimore, unpublished results), converting the virion RNA into the natural mRNA form. Protein synthesis initiation factors were prepared from rabbit reticulocytes as described by Schreier *et al.* (19), through the purification steps indicated in each experiment. Preparation 1 of eIF-4B was purified through step 4 and preparation 2 through step 5 of the procedure described (19).

RESULTS

Selective Inhibition of mRNA Translation. To determine the rate of inhibition of cellular protein synthesis after poliovirus infection, samples of infected cells were pulse labeled for 15-min periods with [³⁵S]methionine at various times after virus infection of HeLa cells. The labeled proteins were then analyzed by gel electrophoresis as shown in Fig. 1A. Poliovirus protein synthesis became evident at 2 hr, and extensive inhibition of host protein synthesis occurred between 2 and 2.5 hr. Quantitation of a host protein band (arrow, Fig. 1A) revealed little decrease in the rate of its synthesis before 2 hr and a decrease to 20% of the original rate between 2 and 2.5 hr. In contrast, synthesis of a poliovirus protein (VP-3) increased 3-fold during this time.

To examine this selective inhibition of translation *in vitro*,

we prepared cell-free translation systems from unlabeled portions of the same cells harvested at 0 time, 1 hr, 2 hr, and 3 hr after virus adsorption. Preparation of the translation systems included a standard preincubation of the extracts at 37° for 45 min (15) and nuclease treatment (16) to eliminate endogenous mRNA translation. The products of translation of VSV mRNAs and poliovirus mRNA in these extracts were then analyzed by gel electrophoresis (Fig. 1B). In extracts prepared from cells immediately after poliovirus infection, VSV mRNA directed the synthesis of four readily detected protein bands (Fig. 1B). From previous work (7) these are known to represent M protein (27,000 daltons), NS protein (45,000 daltons), N protein (53,500 daltons), and the unglycosylated form of G protein (63,500 daltons) running ahead of G protein marker. Compared to the relatively undiminished rate of synthesis in extracts made 1 hr after infection, synthesis of VSV proteins was reduced to less than 20% of the original rate in extracts made 2 hr after infection and was almost completely abolished in 3-hr extracts (see Fig. 1C). In contrast, poliovirus mRNA directed the synthesis of proteins at least 75% as well in extracts made 3 hr after infection as it did in extracts of mock-infected cells. Thus, the *in vitro* translation system reproduces a selective translation inhibition similar to that seen *in vivo*. Note that the inhibition of VSV mRNA translation *in vitro* appears much greater in the 2-hr extract (Fig. 1B) than the inhibition of host protein synthesis at 2 hr *in vivo* (Fig. 1A). This difference is apparently due to the 45-min preincubation of the *in vitro* extracts, because extracts prepared without preincubation showed the major decrease in ability to translate VSV mRNA between 2 and 3 hr after infection (data not shown). Enhanced inhibition due to preincubation is to be expected because, as shown below, a preincubation-dependent inhibition occurs in mixed extracts from poliovirus-infected and uninfected cells.

To determine if the polypeptides synthesized *in vitro* in re-

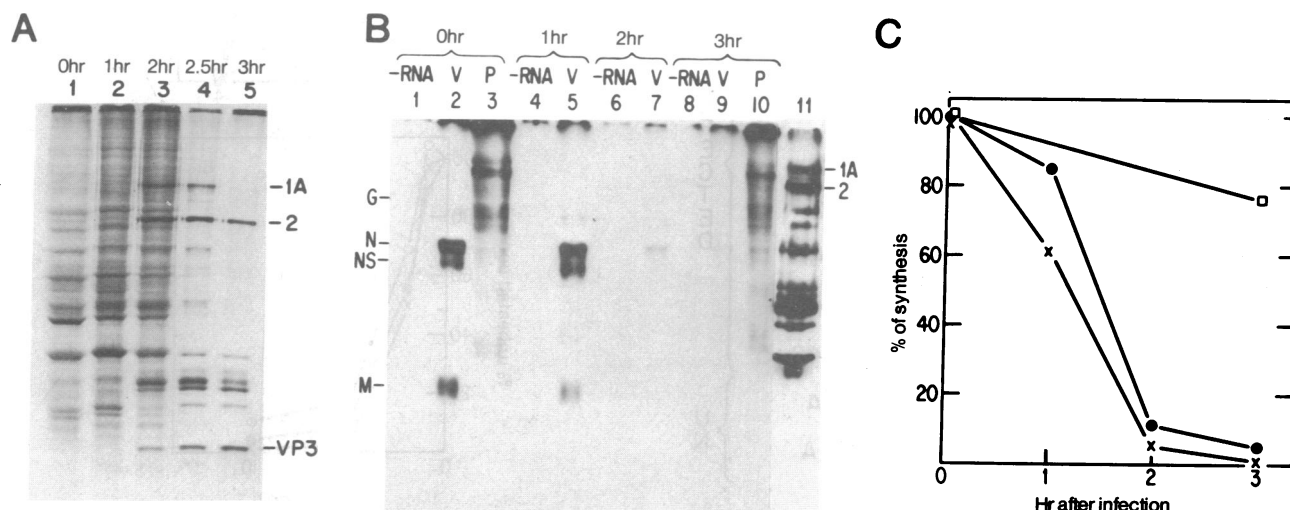


FIG. 1. Kinetics of poliovirus-induced inhibition of translation. (A) Inhibition *in vivo*. HeLa cells were infected with poliovirus and pulse labeled with [³⁵S]methionine (10 μ Ci/ml). Cytoplasmic proteins were subjected to gel electrophoresis (10% acrylamide) and detected by autoradiography. Times given above lanes 1–5 refer to times after virus adsorption at which each 15-min pulse label was begun. The 0 time sample was from cells that were mock-infected. Equal radioactivity was placed into each lane. Total acid-precipitable incorporation (cpm per 0.01 ml of cells) was 1.7×10^4 (0 time), 4.4×10^4 (1 hr), 5.5×10^4 (2 hr), 6.6×10^4 (2.5 hr), and 7.6×10^4 (3 hr). (B) Inhibition *in vitro*. [³⁵S]Methionine-labeled proteins synthesized *in vitro* were subjected to gel electrophoresis (12.5% acrylamide) and detected by fluorography (22). Times above lanes 1–10 indicate time after virus adsorption at which the cells were harvested for preparation of the three translation systems. The 0 time extract was from cells that were mock-infected. Ten microliters of each translation reaction were analyzed: lanes 1, 4, 6, and 8, no added RNA; lanes 2, 5, 7, and 9, VSV mRNA; lanes 3 and 10, poliovirus mRNA. Lane 11 shows marker proteins synthesized *in vivo* 3 hr after poliovirus infection. Positions of NCVP1A and NCVP2 as well as unlabeled VSV virion proteins are indicated. (C) Quantitation of inhibition *in vitro*. Regions of the gel (B) containing proteins synthesized *in vitro* were excised and radioactivity was determined in a scintillation counter. Background radioactivity from the same regions (no added RNA) was subtracted. Radioactivity in N and NS proteins (●) and M protein (×) as well as total poliovirus proteins (□) is plotted as percent of synthesis obtained in the mock-infected extract.

sponse to poliovirus RNA were related to authentic poliovirus proteins, we analyzed their partial proteolytic cleavage products using the method described by Cleveland *et al.* (23). The results indicated that the *in vitro* products in the range of 50,000–105,000 daltons contained peptides that comigrated with those from poliovirus NCVP-1A marker, and only traces of peptides from other poliovirus proteins were seen (data not shown). Thus, the synthesis does represent virus-related products, but, because NCVP-1A is translated from the 5' half of the poliovirus mRNA (24), the majority of the protein synthesis *in vitro* probably terminates in the 5' half of the RNA.

Effects of Specific Initiation Factors. To determine if the inability of the extracts from infected cells to translate VSV mRNA was due to loss of a specific initiation factor, we added purified initiation factors to the *in vitro* translation system derived from infected cells and analyzed the products synthesized in the presence of VSV mRNA (Fig. 2). Quantitation of the gels showed only background levels of VSV mRNA translation (equivalent to no added RNA) in the presence of eIF-1, eIF-3, eIF-4A, eIF-4C, and eIF-5. In contrast, addition of eIF-4B resulted in VSV mRNA translation at a level of 60% compared to the uninfected extract. Also, a very low level of stimulation by eIF-2 (about 5%) was seen. As shown below, eIF-2 stimulates translation in both infected and uninfected lysates. In contrast, the effect of eIF-4B was specific for the infected lysate.

Additional experiments indicated that the activity that restored translation was actually the eIF-4B protein in the purified factor preparation. The activity of eIF-4B is defined by using a reconstituted system that is dependent on eIF-4B (19). To correlate the effect of restoring VSV mRNA translation in infected extracts with the effect on the reconstituted system we used two approaches. First, the elution of the two activities

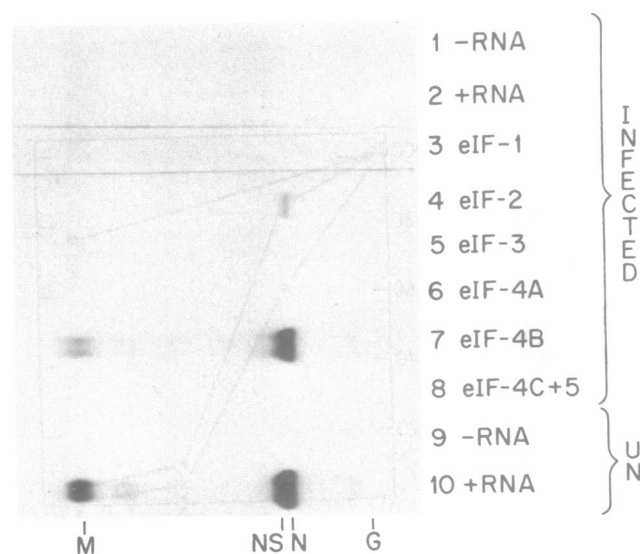


FIG. 2. Effects of initiation factors on VSV mRNA translation. [35 S]Methionine-labeled proteins synthesized *in vitro* were subjected to gel electrophoresis and detected by autoradiography as shown. Lanes 1–8 show products synthesized in a lysate from poliovirus-infected cells; lanes 9 and 10 show products synthesized in a mock-infected lysate. Lanes 1 and 9, no added RNA; lanes 2–8 and 10, plus VSV mRNA and the indicated initiation factors. Positions of unlabeled VSV virion proteins are indicated. Approximate amounts of initiation factors per 25- μ l reaction mixture and the purification steps used (19) were as follows: eIF-1, 0.125 μ g, step 3; eIF-2, 0.5 μ g, step 3; eIF-3, 1.0 μ g, step 4; eIF-4A, 0.75 μ g, step 4; eIF-4B, 1.5 μ g, step 4; eIF-4C + 5, 0.1 μ g, step 3. Electrophoresis was from right to left.

during the final step of purification of eIF-4B was examined. After four purification steps, both activities had been evident, and during the final gradient elution from a phosphocellulose column, the activities coeluted (Fig. 3A). At this stage, eIF-4B was estimated to be 70% pure (19). As a second test, the heat-inactivation kinetics of the two activities were tested. The two activities were lost with indistinguishable kinetics (Fig. 3B).

Effects of Initiation Factors in Infected and Uninfected Lysates. To quantitate the effects of eIF-4B on VSV mRNA and poliovirus mRNA translation in extracts from poliovirus-infected and uninfected cells, we analyzed bands of labeled VSV proteins excised from gels for radioactivity (Table 1). Factor eIF-4B (prep. 1) stimulated VSV (M or NS + N) translation less than 2-fold in the uninfected cell extract, but restored translation in extracts of infected cells from undetectable levels to 50% (N + NS protein) or 100% (M protein) of the level seen in the uninfected lysate. A more highly purified eIF-4B (prep. 2) was less active, but showed some restoration of VSV translation in the infected extract. The effects of eIF-4B on poliovirus mRNA translation were quite different. Prep. 1 inhibited its translation about 3-fold in both lysates, while prep. 2 had little or no effect in either lysate. The inhibition by eIF-4B (prep. 1) may thus have been due to a contaminating protein. Because addition of eIF-2 to infected lysates had given small but detectable

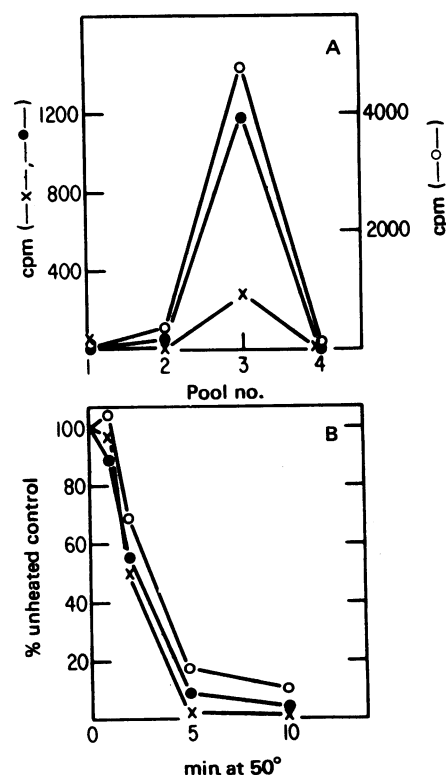


FIG. 3. Purification and heat inactivation of eIF-4B. (A) Phosphocellulose chromatography. Fractions obtained during gradient elution of eIF-4B from phosphocellulose (step 5, ref. 19) were pooled, concentrated, and assayed for eIF-4B activity in the initiation factor-dependent system b (19) and for stimulation of VSV M protein and N + NS protein synthesis in an infected lysate as in Figs. 1C and 2. Pools 1–4 eluted at KCl concentrations of 0.1 M, 0.13–0.23 M, 0.23–0.33 M, and 0.33–4 M, respectively. O, eIF-4B activity; ●, stimulation of N plus NS synthesis; X, stimulation of M synthesis. (B) Heat inactivation. Samples of step 4 eIF-4B in 20 mM Tris-HCl, pH 7.6/100 mM KCl/0.1 mM EDTA/14 mM 2-mercaptoethanol/10% glycerol were incubated at 50° for 1, 2, 5, and 10 min, and assayed as described in A.

Table 1. Effects of eIF-4B and eIF-2 on VSV and poliovirus mRNA translation

Protein	Uninfected extract						Infected extract					
	-RNA	+RNA	eIF-4B		eIF-2*	eIF-2 + eIF-4B (prep. 2)*	-RNA	+RNA	eIF-4B		eIF-2*	eIF-2 + eIF-4B (prep. 2)*
			(prep. 1)*	(prep. 2)*					(prep. 1)*	(prep. 2)*		
N + NS	208	1,440	2,300	2,310	19,100	29,000	158	155	1,100	418	369	4,250
M	63	725	959	1,330	11,000	14,900	67	53	1,150	186	66	2,770
Polio	1,206	32,500	10,700	38,600	101,000	89,500	1,541	24,400	7,210	25,800	58,100	51,000
Globin	79	2,230	ND	ND	ND	ND	62	243	ND	861	ND	ND

Translation of VSV mRNAs, poliovirus mRNA, and rabbit globin mRNA was as described in *Materials and Methods*. The amounts of initiation factors added per 25- μ l reaction mixture were approximately 1.5 μ g (eIF-4B, prep. 1), 0.15 μ g (eIF-4B, prep. 2), and 0.5 μ g (eIF-2, purified through step 3, ref. 19). The proteins synthesized in response to each mRNA were analyzed by gel electrophoresis, and protein bands were quantitated as described in the legend of Fig. 1C. The numbers given are the cpm in the protein bands or in the equivalent region of the gel for translation without RNA. The stimulatory effect of eIF-2 on VSV mRNA translation was variable (4- to 15-fold) in different extracts. ND = not determined.

* In the presence of RNA.

stimulation of VSV protein synthesis in preliminary experiments, we also tested this factor alone and in combination with eIF-4B (prep. 2) in this experiment (Table 1). Addition of eIF-2 resulted in greater than 10-fold stimulation of VSV mRNA translation in uninfected lysates in the presence or absence of eIF-4B. A similar 10-fold increase in VSV translation could be seen in the infected lysate when eIF-2 was assayed in the presence of eIF-4B (prep. 2). Poliovirus mRNA translation was also stimulated by eIF-2 (about 3-fold) in lysates from infected or uninfected cells. These results indicate that eIF-2 can be limiting for translation in both the infected and uninfected lysates but does not appear to be directly involved in the inhibition of translation by poliovirus.

In a separate experiment, translation of globin mRNA in infected and uninfected cell extracts was assayed. The results were qualitatively similar to those obtained with VSV mRNA; some globin synthesis, however, was clearly detectable in the infected lysate. This suggests that all mRNAs may not show the same extent of translation inhibition in infected lysates.

Inactivation of eIF-4B *In Vitro*. To examine the possibility that inactivation of eIF-4B might occur *in vitro*, we measured translation of VSV mRNA in mixed extracts from poliovirus-infected and uninfected cells. The results (Fig. 4) show that VSV mRNA translation occurred in extracts from uninfected and poliovirus-infected cells that had been mixed prior to translation. The level of translation was approximately proportional to the amount of uninfected extract present in the mixture. This result shows that there was not an excess of an inhibitor in the infected lysate that could cause a rapid inactivation of translation. If the mixed extracts were preincubated for 30 or 60 min prior to translation, a loss of ability to translate VSV mRNA was seen (Fig. 4). That this *in vitro* inactivation was due to loss of eIF-4B activity was shown by addition of eIF-4B after a 30-min preincubation of the mixed extract. This addition increased VSV mRNA translation to approximately the unincubated level (Fig. 4). Control experiments showed that the extract from uninfected cells lost only 10% of its activity after 60 min of preincubation (Fig. 4).

DISCUSSION

We have described here an *in vitro* translation system from poliovirus-infected cells that reproduces the selective translation inhibition of host and VSV mRNA translation seen *in vivo*. This inhibition can be overcome by addition of a single purified translation initiation factor, eIF-4B. Also, lysates from poliovirus-infected cells can inactivate eIF-4B when it is added to the lysate (data not shown) or in mixed lysates from infected

and uninfected cells. The mechanism of inactivation is not known, but it is presumably carried out by a viral protein (25).

Because poliovirus mRNA translation occurs normally in lysates from infected cells and is not stimulated by added eIF-4B, it appears to require little or no eIF-4B or can use a modified form of the factor. Factor eIF-4B interacts with the capped 5' end on eukaryotic mRNA (12), and the capped 5' end is important for ribosome recognition of mRNA (9). Poliovirus mRNA lacks a capped 5' end and, therefore, must bypass any 5'-cap-dependent ribosome recognition mechanism. Inactivation of an initiation factor involved in 5'-cap recognition may then favor poliovirus mRNA translation.

Consistent with our work, a previous study has shown that initiation of translation of cellular mRNA is inhibited in extracts from poliovirus-infected cells and can be increased by addition of a ribosomal wash fraction (3). Studies on mengovirus and encephalomyocarditis virus, two other picornaviruses (26, 27), did not reveal consistent selectivity in translation of host and viral mRNAs in extracts from virus-infected and uninfected

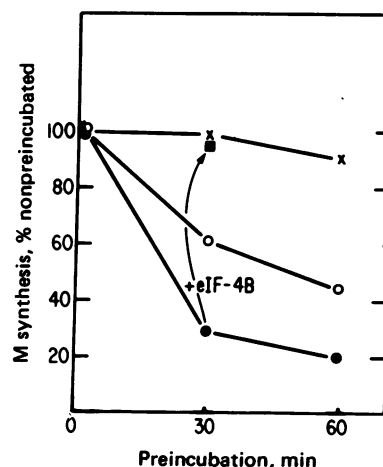


FIG. 4. Translation in mixed lysates. *In vitro* translation was in uninfected lysates or in poliovirus-infected and uninfected lysates mixed at the indicated ratios. Lysates were preincubated for the indicated times prior to addition of translation reaction components and VSV mRNA. VSV M protein synthesis was quantitated as in the legend of Fig. 1C. The synthesis of M protein in the mixture of infected and uninfected lysates (1:1, 30-min preincubation) with eIF-4B (prep. 1, approximately 1.5 μ g) added after preincubation is indicated, X, Uninfected, 551 cpm at 100%; O, uninfected + infected (4:1), 443 cpm at 100%; ●, uninfected + infected (1:1), 225 cpm at 100%.

cells. Another study on encephalomyocarditis virus suggested that the inhibition of host mRNA translation during infection might be explained, at least in part, by direct competition between encephalomyocarditis virus mRNA and cellular mRNAs for initiation factor eIF-4B (28). Our own direct competition experiments have shown that VSV mRNA translation is greatly favored over poliovirus mRNA translation when both types of mRNA are present at equimolar saturating concentrations in lysates from uninfected HeLa cells (data not shown). Thus, the inhibition of VSV mRNA translation by poliovirus clearly requires direct effects on the translation system itself.

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